

Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants

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A reproducible system for the generation of fertile, transgenic maize plants has been developed. Cells from embryogenic maize suspension cultures were transformed with the bacterial gene *bar* using microprojectile bombardment. Transformed calli were selected from the suspension cultures using the herbicide bialaphos. Integration of *bar* and activity of the enzyme phosphinothricin acetyltransferase (PAT) encoded by *bar* were confirmed in all bialaphos-resistant callus lines. Fertile transformed maize plants (R_0) were regenerated, and of 53 progeny (R_1) tested, 29 had PAT activity. All PAT-positive progeny analyzed contained *bar*. Localized application of herbicide to leaves of *bar*-transformed R_0 and R_1 plants resulted in no necrosis, confirming functional activity of PAT in the transgenic plants. Cotransformation experiments were performed using a mixture of two plasmids, one encoding PAT and one containing the nonselected gene encoding β -glucuronidase. R_0 plants regenerated from co-transformed callus expressed both genes. These results describe and confirm the development of a system for introduction of DNA into maize.

INTRODUCTION

Over the past decade, the value of introducing foreign genes into plants has been well documented. In dicotyledonous species, genetic transformation has been used to study plant processes such as the action of transposable genetic elements (Finnegan et al., 1989; Spena et al., 1989), the impact of manipulating metabolic pathways (Last and Gray, 1990; van der Krol et al., 1990), tissue-specific gene expression (Stockhaus et al., 1987; Benfey and Chua, 1989), as well as other aspects of gene regulation (Matzke et al., 1989; Rocha-Sosa et al., 1989). The application of genetic transformation techniques to answer basic research questions will increase in frequency and sophistication as methods continue to improve. In addition, introduction of potentially useful agronomic traits such as insect, viral, and herbicide resistance has been accomplished in a number of dicotyledonous crop species (Gasser and Fraley, 1989). For example, transgenic tobacco and tomato producing modified *Bacillus thuringiensis* toxin display enhanced resistance to lepidopteran pests (Fischhoff et al., 1987; Vaeck et al., 1987). Tomato transformed with the tobacco mosaic virus coat protein gene proved resistant to viral infection in both greenhouse and field tests (Nelson et al., 1988). Genes conferring resistance to a number of herbicides have also been introduced into

various dicotyledonous crop species (Comai et al., 1985; De Block et al., 1987; Haughn et al., 1988). Extending transformation techniques to monocotyledonous plants, in particular the Gramineae, is of great importance for applied as well as basic research because members of this family (e.g., maize, rice, and wheat) constitute a substantial portion of the world's food crop.

Despite prolonged and substantial effort by many laboratories, development of genetic transformation techniques for the major cereal crops has been slow. The recalcitrance of maize and most other monocot species to *Agrobacterium*-mediated transformation has led to the development of direct DNA delivery methods. Electroporation of maize protoplasts (Fromm et al., 1986) has produced transgenic plants (Rhodes et al., 1988); however, these plants were not fertile. Despite published success in regenerating fertile maize plants from protoplasts (Prioli and Sondahl, 1989; Shillito et al., 1989), regeneration of fertile, transgenic maize plants has not been reported. Presently, rice is the only cereal grain for which a protoplast-based transformation system has been developed that can produce fertile, transgenic plants (Shimamoto et al., 1989).

The problems surrounding cereal transformation have been difficult to elucidate. A number of potential impediments to successful monocot transformation have been suggested (Potrykus, 1989). For cell cultures, these in-

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clude the lack of DNA delivery and selection methods that are compatible with regeneration of fertile plants.

Recently, we have been able to overcome these obstacles by bombarding embryogenic maize suspension cells with tungsten particles coated with the selectable marker gene *bar*. The *Streptomyces hygroscopicus bar* gene encodes phosphinothricin acetyltransferase (PAT), an enzyme that inactivates the herbicidal compound phosphinothricin (PPT) by acetylation (Murakami et al., 1986; Thompson et al., 1987). PPT inhibits glutamine synthetase (Tachibana et al., 1986a), causing rapid accumulation of ammonia and cell death (Tachibana et al., 1986b). This gene has been used in *Agrobacterium*-mediated transformation of tobacco, tomato, potato (De Block et al., 1987), and rape (De Block et al., 1989). The resulting transgenic plants were protected against the broad-spectrum herbicides glufosinate (the ammonium salt of PPT) and bialaphos (a compound containing PPT). Previously, we used *bar* as a selectable marker for the identification of nonembryogenic Black Mexican Sweet (BMS) maize cells transformed by microprojectile bombardment (Spencer et al., 1990). Here we describe bialaphos selection of stable transformants from various embryogenic maize suspension cultures of A188 \times B73 and A188 \times B84, including cryopreserved suspension cultures. Fertile transgenic plants were regenerated from transformed embryogenic callus lines, and transgenic R_0 and R_1 plants produced sufficient functionally active PAT to protect leaf tissue from the herbicidal action of PPT.

RESULTS

Bombardment and Selection

Cells from embryogenic maize suspension cultures were bombarded with the *bar*-containing plasmid pDPG165 alone or in combination with a plasmid encoding β -glucuronidase (GUS), pDPG208. Schematic maps of these plasmids are shown in Figure 1. In selection experiments in which a GUS plasmid was included, two of the filters with bombarded cells were histochemically stained to determine the number of foci (clusters of transiently expressing cells) with GUS activity 48 hr post-bombardment. The total number of foci was at least 1000 per filter in all experiments. In two separate experiments designed to quantitate transiently expressing cells (using an A188 \times B73 suspension culture designated SC82), the mean number and standard deviation of GUS-staining foci per filter was 1472 ± 211 and 2930 ± 390 ($n = 3$ and 4 , respectively). The number of cells in a focus ranged from one to 10 and averaged two to three. In these experiments the numbers of stained foci were at least fivefold greater than in previous experiments using BMS suspension cells (Spencer et al., 1990). This difference is in part attributed to modifications

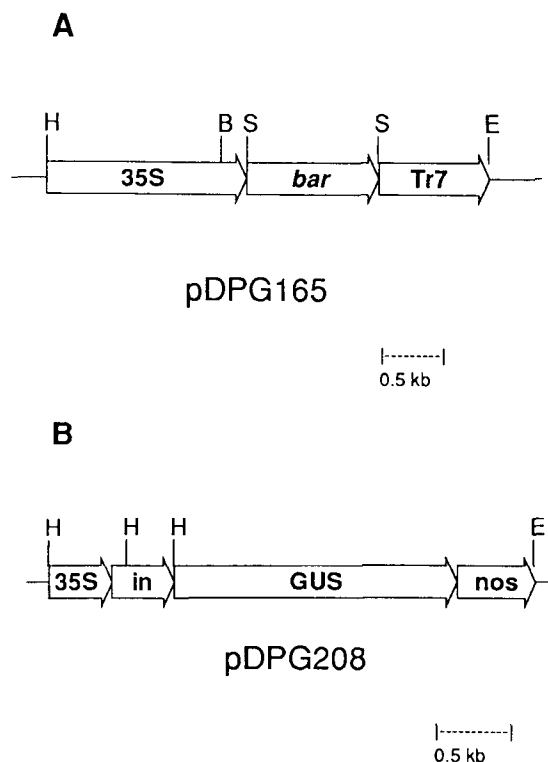


Figure 1. Schematic Representation of Plasmids Used in Bombardment Experiments.

(A) pDPG165.

(B) pDPG208.

E, EcoRI; H, HindIII; B, BamHI; S, SmaI.

in the bombardment procedure, such as the use of screens to disperse tungsten particles and modifications in the precipitation protocol.

After bombardment, cells on filters were resuspended in nonselective liquid medium, cultured for 1 to 2 weeks, and transferred to filters overlaying solid medium containing 1 or 3 mg/L bialaphos. The degree of inhibition of tissue growth during selection was dependent upon the distribution of the cells on the filter and on the concentration of bialaphos used. At the density plated [0.5 mL packed cell volume (PCV)/filter], the growth of the cells cultured on 1 mg/L bialaphos was only partially inhibited (30% to 50% of nonselected growth as determined by visual observation). After 3 to 4 weeks, much of this tissue was transferred as discrete clumps (~5 mm in diameter) to identical medium. On medium containing 3 mg/L bialaphos, the growth of cells on the original selection filter was severely inhibited (~10% of nonselected growth) and selection was continued without removing the tissue from the original filter. Using either selection protocol (1 or 3 mg/L bialaphos), resistant cell colonies emerged on the selection plates approximately 6 to 7 weeks after bombardment, as

shown in Figure 2A. No resistant colonies were recovered from selection plates containing nonbombarded tissue. Bialaphos-resistant callus lines were maintained and expanded on selection medium and much of this tissue was embryogenic, as shown in Figure 2B. Observed growth of selected callus lines maintained on bialaphos was similar to that of control tissue on nonselective medium.

Analysis of Transformants

The suspension culture used in initial experiments, SC82, was derived from embryogenic type II callus of A188 ×

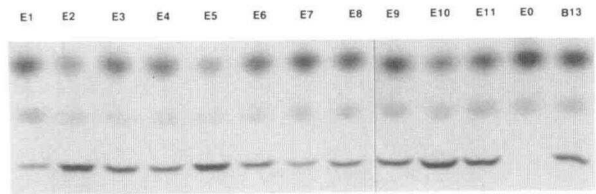


Figure 3. PAT Activity in Embryogenic SC82 Transformants E1 to E11 and a Nonselected Control (E0).

Twenty-five micrograms of protein extract were loaded per lane. B13 is a BMS-*bar* transformant (see Spencer et al., 1990).

B73. Bialaphos-resistant callus lines selected from SC82 were analyzed for activity of the *bar* gene product PAT by thin-layer chromatography. Protein extracts from 11 callus lines (E1 to E11) contained PAT activity, as shown in Figure 3, and activity levels varied approximately 10-fold among the isolates. Isolates E1 to E11 were further analyzed for the presence of *bar*. Chromosomal DNA from E1 to E11 was digested with EcoRI and HindIII to release the 1.9-kb *bar* expression unit (35S promoter-*bar*-Tr7 3' end, Figure 1A). Genomic DNA from all 11 bialaphos-resistant isolates contained *bar*-hybridizing sequences, as shown in Figure 4. The hybridization in all isolates to a fragment migrating slightly larger than 2 kb may be due to contaminating pUC19 sequences contained in this *bar* probe preparation; no such hybridization occurred in subsequent experiments using the same genomic DNA and a different preparation of the *bar* probe. Hybridization to a 1.9-kb fragment in eight of the 11 isolates indicated that these isolates contained intact copies of the 1.9-kb *bar* expression unit. The estimated copy numbers of the intact unit ranged from one or two (E1, E7, E8, E10, and E11) to approximately 20 (E3, E4, and E6). Hybridization with the *bar* probe in isolates E2 and E5 occurred only to a single, higher molecular weight fragment (~3 kb). To establish that the PAT structural gene was intact in these isolates, genomic DNA was digested with SmaI, which releases a 559-bp fragment containing the PAT structural gene (Figure 1A), and hybridized to ³²P-labeled *bar*. This analysis confirmed the presence of a single intact copy of *bar* (data not shown).

The hybridization patterns of some of the SC82 isolates were identical (E2 and E5; E7 and E8; and E3, E4, and E6); therefore, it is probable that some isolates did not arise from independent transformation events but represent transformants that were separated during selection. Seven hybridization patterns were unique, likely representing seven independent single-cell transformation events. The patterns and intensities of *bar* hybridization for the seven transformants were unchanged during 4 months in culture (data not shown), providing evidence for the stability of the integrated sequences. The seven independent transformants (E1, E2/E5, E3/E4/E6, E7/E8, E9,

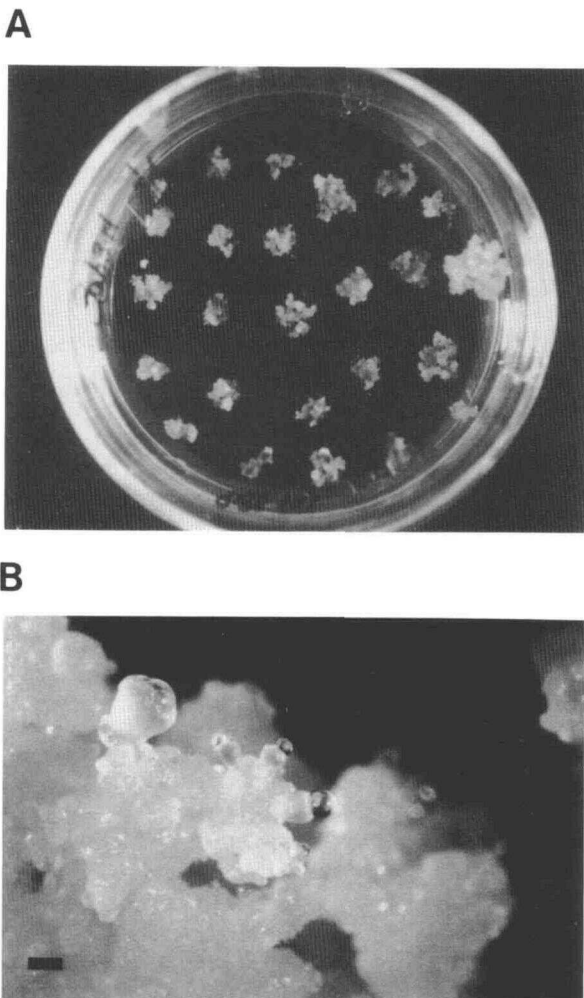


Figure 2. Bialaphos Selection of Embryogenic Callus.

(A) SC82 bialaphos-resistant colony selected on 1 mg/L bialaphos (7 weeks post-bombardment).

(B) Embryogenic SC82 bialaphos-resistant callus selected and maintained on 1 mg/L bialaphos (bar = 1 mm).

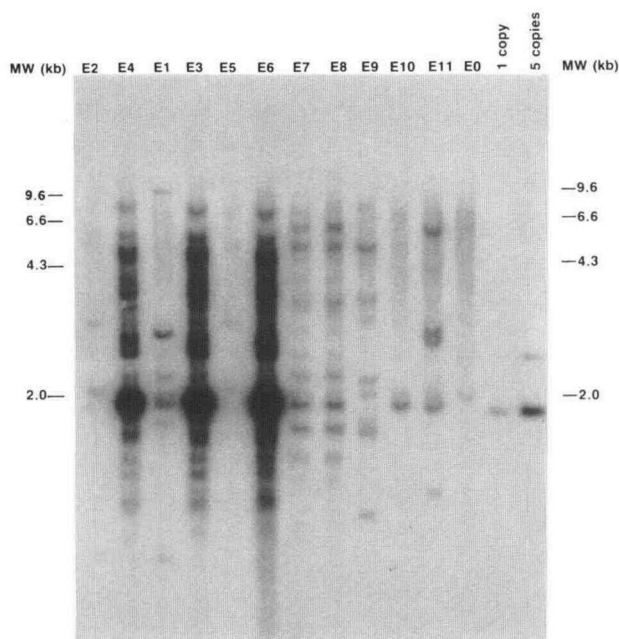


Figure 4. Integration of the *bar* Gene in Bialaphos-Resistant SC82 Isolates E1 to E11.

DNA gel blot of genomic DNA (4 μ g/lane) from E1 to E11 and a nonselected control (E0) digested with *Eco*RI and *Hind*III. Blot was hybridized with 32 P-labeled *bar* from pDPG165 (25 \times 10⁶ Cerenkov cpm). Lanes designated 1 copy and 5 copies refer to the diploid genome and contain 1.9 pg and 9.5 pg, respectively, of the 1.9-kb *bar* expression unit released from pDPG165 with *Eco*RI and *Hind*III.

E10, and E11) were derived from two separate bombardment experiments (1 and 2) and a total of 10 bombarded filters. These data are summarized in Table 1.

Bombardment experiments were also performed using a suspension culture, SC716, derived from a separate initiation of type II callus of A188 \times B73. The SC716 suspension culture was cryopreserved 5 months after the suspension was initiated and then reestablished after 8 months' storage in liquid nitrogen before use in bombardment experiments. The SC716 cells were bombarded with separate plasmids encoding PAT (pDPG165) and GUS (pDPG208). Selection was as described for SC82, using 1 mg/L bialaphos. Twenty-one bialaphos-resistant callus lines were isolated; all expressed PAT at levels comparable with those of the SC82 isolates (data not shown). DNA gel blot analyses of the SC716 isolates demonstrated that all bialaphos-resistant isolates tested (R1 to R21) had *bar*-hybridizing sequences, as shown in Figure 5A. Thirteen isolates contained intact copies of the 1.9-kb *bar* expression unit. Twenty unique hybridization patterns were observed among the 21 SC716 isolates (R8 and R10 were identical), suggesting 20 independent transformation

events. These 20 transformants were from two experiments (4 and 5) and a total of 16 bombarded filters (Table 1).

Using other embryogenic suspension cultures, an SC82 culture that was reinitiated from cryopreserved cells (experiment 6) or an A188 \times B84 (SC94) suspension culture (experiment 3), numerous independent transformants were recovered (19 and 8, respectively; Table 1). All transformants contained the *bar* gene and expressed PAT. The copy number of *bar*-hybridizing sequences, the diversity of *bar*-hybridizing patterns, and levels of PAT expression (data not shown) were comparable with the experiments described above and with those performed previously with BMS (Spencer et al., 1990).

Cotransformation

Transformants from SC716 experiments (4 and 5) were analyzed for integration and expression of the nonselected gene encoding GUS. As determined by histochemical assay, four of the SC716 transformants (R5, R7, R16, and R21) had detectable GUS activity 3 months post-bombardment (Table 1). Expression patterns observed in the four coexpressing callus lines varied. The number of cells with GUS activity within any given transformant sampled ranged from ~5% to ~90% and, in addition, the level of GUS activity in those cells varied. Cointegration frequency was determined by washing the genomic blot hybridized with *bar* (Figure 5A) and probing with 32 P-labeled GUS-coding sequence. *Eco*RI and *Hind*III, which excised the *bar* expression unit from pDPG165, also released from pDPG208 a 2.1-kb fragment containing the GUS-coding sequence and the *nos* 3' end (Figure 1B). Seventeen of the independent *bar* transformants contained sequences that hybridized to the GUS probe; R2, R14, and R19 did not (Figure 5B; Table 1). Transformants in which GUS activity was detected (R5, R7, R16, and R21) contained the 2.1-kb *Eco*RI/*Hind*III fragment encoding GUS (Figure 5B). Transformants that contained large numbers of *bar*-hybridizing fragments (R1, R5, and R21) also contained large numbers of fragments that hybridized to the gene encoding GUS (Figures 5A and 5B). Similar observations using independent plasmids were reported using PEG-mediated transformation of A188 \times BMS protoplasts (Lyznik et al., 1989) and bombardment-mediated transformation of BMS suspension cells (Spencer et al., 1990).

Cotransformation analyses were also performed on 19 independent bialaphos-resistant isolates selected from a suspension culture reinitiated from cryopreserved SC82 cells (experiment 6, Table 1). The frequency of cointegration and coexpression in these isolates was similar to that described for SC716 isolates (Table 1), as was the heterogeneous pattern of GUS staining. For example, one transformant, Y13, which contained intact GUS coding sequence (data not shown), exhibited chimeric GUS activity

Table 1. Summary of Maize Transformation Experiments

Exp.	Culture Bombarded	Filters Bombarded	Independent <i>bar</i> Transformants Recovered	No. with Intact <i>bar</i> Expression Units	No. with GUS Coding Sequence	No. with GUS Activity	Cointegration Frequency (%)	Coexpression Frequency (%)
1	SC82	4	4	3	NA ^a			
2	SC82	6	3	2	NA			
3	SC94	10	8	6	NA			
4	SC716 ^b	8	13	8	11	3	85	23
5	SC716 ^b	8	7	4	6	1	86	14
6	SC82 ^b	4	19	17	13	3	68	16
Totals		40	54	40	30	7	77 (30/39)	18 (7/39)

^a NA, not applicable; only pDPG165 DNA used or cotransformation analysis not done.

^b Culture reinitiated from cryopreserved cells.

in callus tissue, as shown in Figure 6. This type of expression pattern has been described previously in cotransformed BMS cells (Klein et al., 1989; Spencer et al., 1990). Variable activity detected in the cells from a single transformant may be attributed to differential expression of the genes, absence of the gene in some cells, or variable penetration of the GUS substrate.

Regeneration and Analysis of R₀ Plants

Using several regeneration protocols, a total of 76 plants were regenerated from four of the SC82 transformants selected in experiments 1 and 2; 73 plants reached maturity. A total of 219 plants were regenerated from 10 SC716 transformants selected in experiments 4 and 5; to date, 35 have reached maturity. Regeneration from callus lines selected in other experiments is in progress. A summary of regeneration efforts is presented in Table 2.

Twenty-one R₀ plants representing each of four regenerable, transformed SC82 callus lines (E2/E5, E3/E4/E6, E10, and E11) selected in experiments 1 and 2 were analyzed for PAT activity. Protein extracts from leaves of each of these plants contained PAT activity, and the activity levels were comparable with levels in callus lines from which the plants were regenerated (data not shown). The activity in extracts from one plant regenerated from each callus line is shown in Figure 7. Genomic DNA from these four callus lines and two R₀ plants derived from each callus line was analyzed for the presence of *bar*, as shown in Figure 8. All R₀ plants analyzed contained sequences that hybridized to *bar*. Furthermore, in all instances the hybridization profiles observed in plant DNA were identical in pattern and intensity to the hybridization profiles of the corresponding callus DNA. To confirm that the *bar*-hybridizing sequences were integrated into chromosomal DNA, undigested genomic DNA from one plant derived from

each independent transformant was analyzed by DNA gel blot hybridization. Hybridization to *bar* was observed only in high molecular weight DNA (data not shown).

Analysis of R₀ plants regenerated from SC716 transformants is not complete; however, two plants from each of the 10 regenerable SC716 callus lines have been tested for PAT activity (data not shown). For seven SC716 callus lines both plants contained PAT activity; for three callus lines one plant was PAT-positive, the other was PAT-negative. The reason for the lack of PAT activity in some SC716 plants has not yet been determined but may be due to inactivation of, or the absence of, *bar* in some plants.

Phenotypes of R₀ Plants

Plants regenerated from SC82 transformants (E2/E5, E3/E4/E6, E10, and E11) were similar to seed-derived A188 × B73 plants in that they were comparable in height with A188 (3 feet to 5 feet) and exhibited some characteristic B73 traits such as upright leaves and anthocyanin accumulation in stalks and prop roots. Several abnormal phenotypic characteristics were common to many SC82 R₀ plants, such as palmate leaves, split leaves, wilting, coarse silk, and underdeveloped tassels. Nontransformed control plants were not regenerated from this culture and, therefore, cannot be compared phenotypically. Pistillate flowers developed on tassels of one E11 (1/6), several E10 (3/22), and almost one-third of the E2/E5 (12/37) plants with a range of three to approximately 20 ovules per tassel. Primary and secondary ears developed frequently on most E2/E5, E10, and E11 plants; a mature E2/E5 plant is shown in Figure 9A. Anthers rarely extruded from the tassels of plants regenerated from SC82 transformants, and the limited number of anthers which were extruded did not dehisce pollen. Some phenotypic characteristics

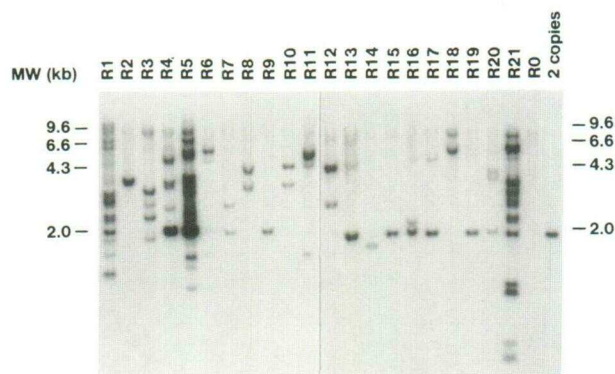
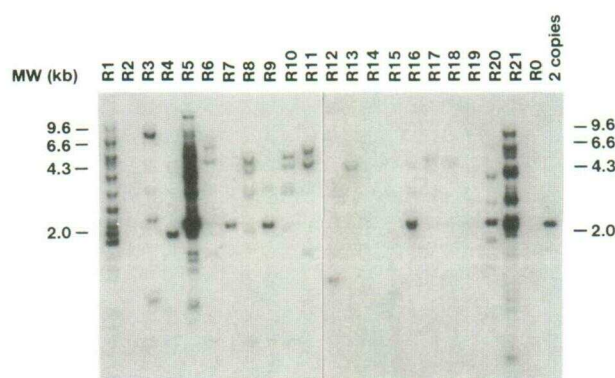
A**B**

Figure 5. Integration of Introduced Genes in Bialaphos-Resistant SC716 Isolates R1 to R21.

(A) DNA gel blot of genomic DNA (6 μ g/lane) from isolates R1 to R21 digested with EcoRI and HindIII and hybridized to 32 P-labeled *bar* probe (10×10^6 Cerenkov cpm). Two copies of the *bar* expression unit per diploid genome is 5.7 pg of the 1.9-kb EcoRI/HindIII fragment from pDPG165.

(B) Blot from (A) was washed to remove the *bar* probe and hybridized with 32 P-labeled GUS probe (35×10^6 Cerenkov cpm). Two copies of the 2.1-kb GUS-containing EcoRI/HindIII fragment from pDPG208 is 6.3 pg.

observed were unique to plants regenerated from a specific callus line, such as the lack of ears on E3/E4/E6 plants and a "grassy" phenotype (up to 21 long, narrow leaves) exhibited by all E11 plants. All SC82 plants senesced prematurely; leaf necrosis began approximately 2 weeks after anthesis.

Of 219 plants regenerated from 10 independent SC716 transformants, approximately 35 have reached maturity (Table 2). The SC716 plants did not exhibit the phenotypic

differences that characterized the individual callus lines of SC82. These plants were more uniform and abnormalities less frequent. The phenotype of these plants closely resembled that of control plants regenerated from an SC716 cryopreserved culture that was not bombarded. Plant height ranged from 3 feet to 6 feet, with the majority of the plants between 5 feet and 6 feet. Most mature plants produced large, multibranched tassels and primary and secondary ears. Pistillate flowers also developed on tassels of several SC716 plants. Although anther extrusion occurred at approximately the same low frequency as in the SC82 plants, a small amount of pollen dehiscence from some extruded anthers. For most of the SC716 plants that reached maturity, senescence did not commence until at least 30 days after anthesis.

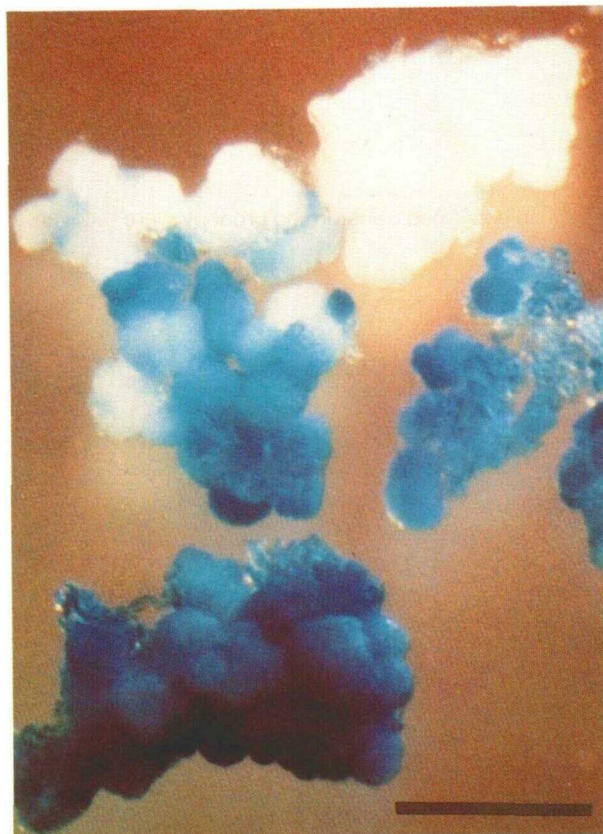


Figure 6. Histochemical Determination of GUS Activity in Bialaphos-Resistant Callus Line Y13.

Y13 was selected from SC82 reinitiated from cryopreserved cells (experiment 6) and tested for GUS activity 3 months post-bombardment. Bar = 0.5 mm.

Table 2. Regenerated Plants (R_0) and Progeny (R_1)

Exp.	Culture Bombarded	Independent <i>bar</i> Transformants Recovered	Regenerable Transformed Callus Lines	R_0 Plants	No. Reaching Maturity	No. of R_0 Producing Kernels	Kernels Recovered	R_1 Plants
1,2	SC82	7	4	76	73	28	184	50
4,5	SC716	20	10	219	(35)	(9)	(51)	(31)
3	SC94	8	2 ^a	11 ^a	(0)	(0)	(0)	(0)
6	SC82	19	4 ^a	23 ^a	(0)	(0)	(0)	(0)

Parentheses indicate that experiment is still in progress, data are still being collected.

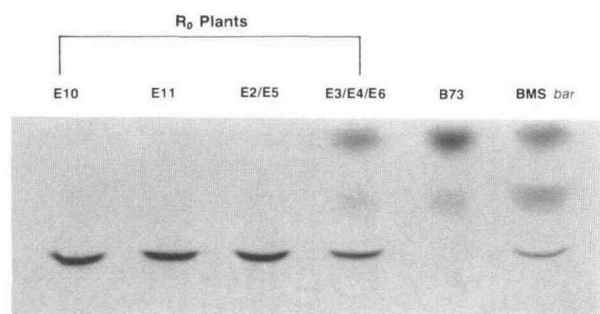
^a Regeneration in progress.

Back-Crossing Transgenic R_0 Plants

Progeny (R_1) were obtained from transformed R_0 plants by back-crossing transformed SC82 plants with seed-derived B73 plants. Anthers that extruded on the tassels of 10 E2/E5 and one E10 R_0 plants were collected, macerated to release pollen, and applied to nontransformed, seed-derived B73 silk. Examination of pollen removed from extruding anthers of SC82 plants revealed that only a small percentage had reached the binucleate stage. Mature, starch-filled pollen grains were rarely observed and failed to germinate when placed on germination medium (Pfahler, 1967). No confirmed transformed progeny were recovered from back-crossing SC82 R_0 pollen with B73 plants. Pollination of transgenic SC82 R_0 ears with nontransformed B73 pollen, however, did result in kernel development. In addition, several kernels developed from pistillate flowers on male inflorescences of SC82 plants that were pollinated

with nontransformed B73 pollen. Kernels on transformed SC82 R_0 plants developed normally for approximately 10 days to 14 days post-pollination. After this period, which corresponded to the initiation of premature senescence in most of the plants, kernels ceased development and often collapsed. A total of 184 kernels developed on the SC82 R_0 plants (Table 2): 21 of 37 E2/E5 plants, five of 22 E10 plants, and two of six E11 plants. Viable progeny were recovered by embryo rescue from 12 E2/E5 plants and one E10 plant. Embryos were excised from kernels that developed on SC82 R_0 plants and ranged in length from about 0.5 mm to 4 mm. Fifty viable progeny have thus far been recovered from SC82 R_0 plants (Table 2); two of which are shown in Figure 9B.

SC716 R_0 plants were also back-crossed with seed-derived B73 plants. To date, from the 35 mature SC716 R_0 plants, nine plants (representing five independent callus lines) yielded 51 kernels, 31 of which produced vigorous R_1 seedlings (Table 2). Most kernels that developed on SC716 plants did not require embryo rescue. Kernels often developed for 30 days to 40 days on the plant and some were germinated in soil. The remaining seed was germinated on MS-based medium (Murashige and Skoog, 1962) to monitor germination and transferred to soil after a few days. In addition to the improved kernel development observed on SC716 R_0 plants relative to SC82 R_0 plants, pollen dehiscence from anthers of several SC716 plants and some of this pollen germinated in vitro (Pfahler, 1967). Ears treated with this pollen have not yet reached maturity.

**Figure 7.** PAT Activity in R_0 Plants.

One plant derived from each of the four transformed regenerable callus lines (E10, E11, E2/E5, and E3/E4/E6) was tested for PAT activity. Protein extracts from a nontransformed B73 plant and a BMS cell culture transformant (Spencer et al., 1990) were included as controls. Approximately 50 μ g of total protein was used per reaction.

Analysis of Progeny

To date, a total of 36 SC82 E2/E5 progeny have been tested for PAT activity and 18 were positive. Of the 10 progeny analyzed for PAT activity in Figure 10A, six were positive. Genomic DNA from these 10 progeny was analyzed by DNA gel blot hybridization for the presence of *bar*, as shown in Figure 10B. The six progeny that had

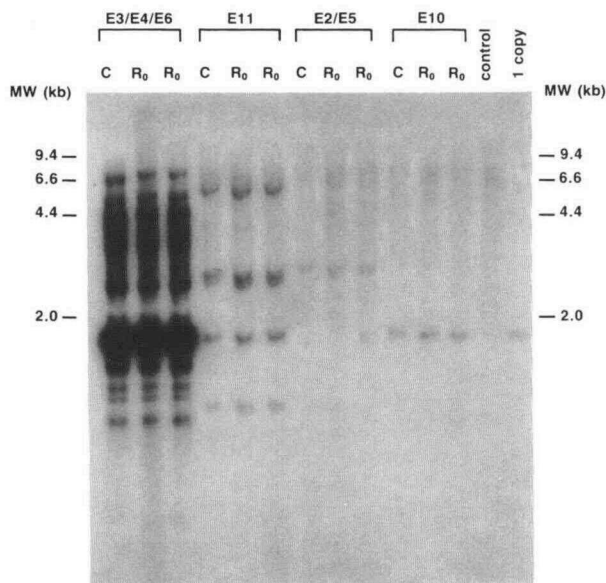


Figure 8. Comparison of *bar* Sequences in Transformed Callus and Corresponding R_0 Plants.

Genomic DNA was digested with EcoRI and HindIII, which release the 1.9-kb *bar* expression unit from pDPG165. Hybridization was to a 559-bp 32 P-labeled SmaI DNA fragment containing *bar* (42×10^6 Cerenkov cpm). The DNA fragments were separated by gel electrophoresis, blotted to nylon membrane, and hybridized to *bar*. Lanes designated E3/E4/E6, E11, E2/E5, and E10 contained 5 μ g of either callus (C) or R_0 plant (R_0) DNA. Control lane contained 5 μ g of DNA from a nontransformed A188 \times B73 plant. Lane designated 1 copy contained 2.3 pg of the 1.9-kb EcoRI/HindIII fragment from pDPG165, representing one copy per diploid genome.

PAT activity each contained a single copy of *bar* identical in mobility to that detected in E2/E5 callus and R_0 plants; the four PAT-negative progeny did not contain *bar*-hybridizing sequences. The ratio of PAT-positive to PAT-negative progeny of E2/E5 R_0 plants is consistent with a 1:1 segregation ratio of a dominant, single-copy gene. The single progeny recovered from an E10 R_0 plant contained PAT activity (data not shown). Segregation of *bar* did not correlate with the phenotypic variability of R_1 plants, such as plant height and tassel morphology. In Figure 9B, the plant on the right contains *bar*, the plant on the left does not. In addition, most of the R_1 plants were more vigorous than the R_0 plants.

Of the 31 R_1 seedlings recovered to date from the SC716 transformants, 10 of 16 tested had PAT activity. PAT activity was detected in four of 10 progeny from R_0 plants representing callus line R18 and six of six progeny from R_0 plants representing callus line R9 (data not shown).

Functional Activity of PAT

Functional activity of PAT was assessed by localized application of a commercial herbicide formulation containing PPT to leaves of SC82 R_0 and R_1 plants. No necrosis was observed on leaves of R_0 plants containing either high levels (E2/E5) or low levels (E3/E4/E6) of PAT. Herbicide-treated E3/E4/E6 and control leaves are shown in Figure 11A. Herbicide was also applied to leaves of E2/E5 progeny segregating for *bar*. As demonstrated in Figure 11B, leaves of R_1 plants expressing *bar* exhibited no necrosis 6 days after application of the herbicide, whereas R_1 plants without *bar* developed necrotic lesions. No necrosis was observed on transformed leaves up to 30 days post-application.

Coexpression of a Nonselected Gene

Plants were regenerated from the coexpressing callus line Y13, shown in Figure 6. Plants regenerated from Y13 (experiment 6, Table 2) were assayed for GUS activity, and histochemically stained leaf tissue from one plant is shown in Figure 12. Numerous cell types including epidermal, guard, mesophyll, and bundle sheath cells stained positive for GUS activity. Staining intensity was greatest in the vascular bundles (data not shown). Although all leaf samples from the regenerated plants tested (5/5) expressed the nonselected gene, some nonexpressing leaf sectors were also observed. Leaf tissue extracts from three Y13 and three control plants were also assayed for GUS activity by fluorometric analysis (Jefferson, 1987). Activity detected in two opposing leaves from each of three Y13 plants tested was at least 100-fold higher than that in control leaves (data not shown).

DISCUSSION

The generation of stably transformed fertile plants is crucial for both the study of basic plant processes and the introduction of new traits into crop species. For maize, the utility of protoplast transformation methods has been limited to monitoring transient expression of introduced genes (Fromm et al., 1986), such as studying aspects of tissue-specific expression (Lee et al., 1989). Recovery of transformed fertile maize plants from electroporated protoplasts has not been documented despite independent reports of infertile protoplast-derived transgenic plants (Rhodes et al., 1988) and of regeneration of nontransformed fertile plants from protoplasts (Prioli and Sondahl, 1989; Shillito et al., 1989). Various alternatives to protoplast transformation have been suggested, such as microinjection (Potrykus, 1989) and laser-mediated DNA delivery into intact



Figure 9. Mature R_0 Plant and R_1 Progeny.

(A) Mature transgenic plant regenerated from E2/E5 callus.
 (B) R_1 progeny derived from E2/E5 plant by embryo rescue.

cells (Weber et al., 1987), but the most rapid progress has been made using microprojectile bombardment (Klein et al., 1989; Spencer et al., 1990).

Microprojectile Bombardment of Maize

Given the goal of obtaining fertile, transgenic maize plants, there are several attractive cellular targets for microprojectile bombardment-mediated DNA delivery. One of these is pollen. Although transient expression of a reporter gene was detected in bombarded tobacco pollen (Twell et al., 1989), stable transformation by microprojectile bombardment of pollen has not yet been reported for any plant species. A second potential target is meristematic tissue. Bombardment of soybean apical meristems with DNA-coated gold particles resulted in chimeric plants containing transgenic sectors; progeny containing the introduced

gene were obtained at a low frequency (McCabe et al., 1988). Using anthocyanin biosynthesis as a visual marker, bombardment of shoot meristems of immature maize embryos with a regulatory gene resulted in sectors of transformed, pigmented tissue (Tomes, 1990). Analysis of cell lineage patterns in maize (McDaniel and Poethig, 1989), however, suggests that germline transformation of maize by such an approach might be difficult. A third target for microprojectile bombardment is cultured cells. Transformants were isolated after bombardment of a nonembryogenic, nonregenerable BMS maize suspension culture, using *neo* and kanamycin selection (Klein et al., 1989) and *bar* and bialaphos selection (Spencer et al., 1990).

Although microprojectile bombardment obviates protoplast isolation, another difficulty in cereal transformation has been the lack of an effective selective agent for totipotent cultures (Potrykus, 1989). Despite the successful use of kanamycin as a selective agent for bombarded

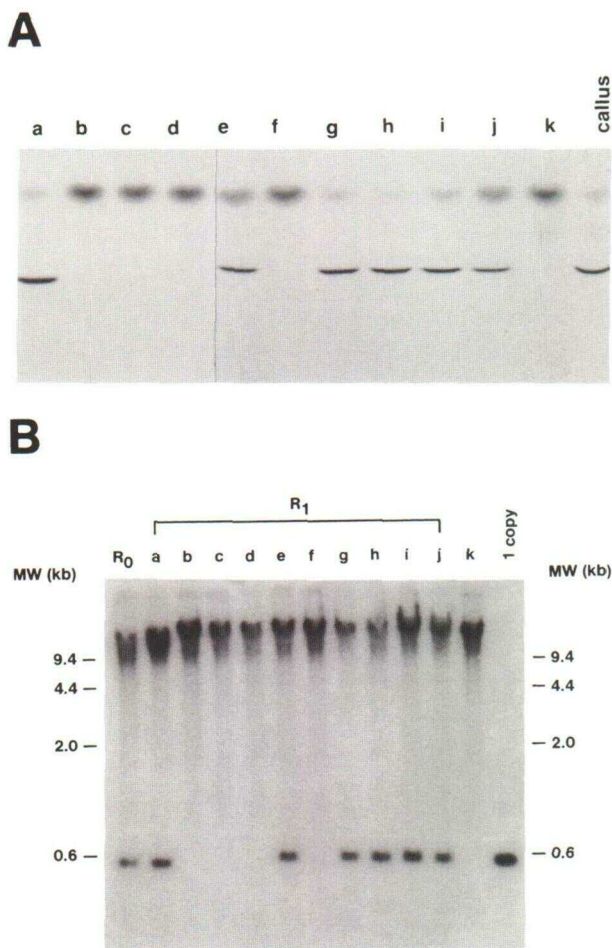


Figure 10. PAT Activity and DNA Gel Blot Analysis of Segregating Progeny from Four E2/E5 R₀ Plants.

(A) Analysis of PAT activity in 10 progeny (lanes a to j) and a nontransformed control plant (lane k). Lanes designated a, b to h, i, and j contained protein extracts from progeny of four separate parental R₀ plants. Lane designated callus contained protein extract from E2/E5 callus. Approximately 25 μ g of total protein per reaction.

(B) DNA gel blot analysis of genomic DNA isolated from the 10 progeny analyzed in **(A)**. Genomic DNA (5 μ g/lane) was digested with SmaI, which releases a 0.6-kb fragment containing *bar* from pDPG165, and hybridized with *bar* probe (50×10^6 Cerenkov cpm). Lane designated R₀ contained DNA from the R₀ parent of progeny a. Lane designated 1 copy contained pDPG165 digested with SmaI to represent approximately 1 copy of the 559-bp fragment per diploid genome (0.8 pg).

nonembryogenic BMS cultures (Klein et al., 1989) and in protoplast selection protocols (Rhodes et al., 1988), its use with cultured maize cells has been hampered by endogenous resistance (Hauptmann et al., 1988; W.J.

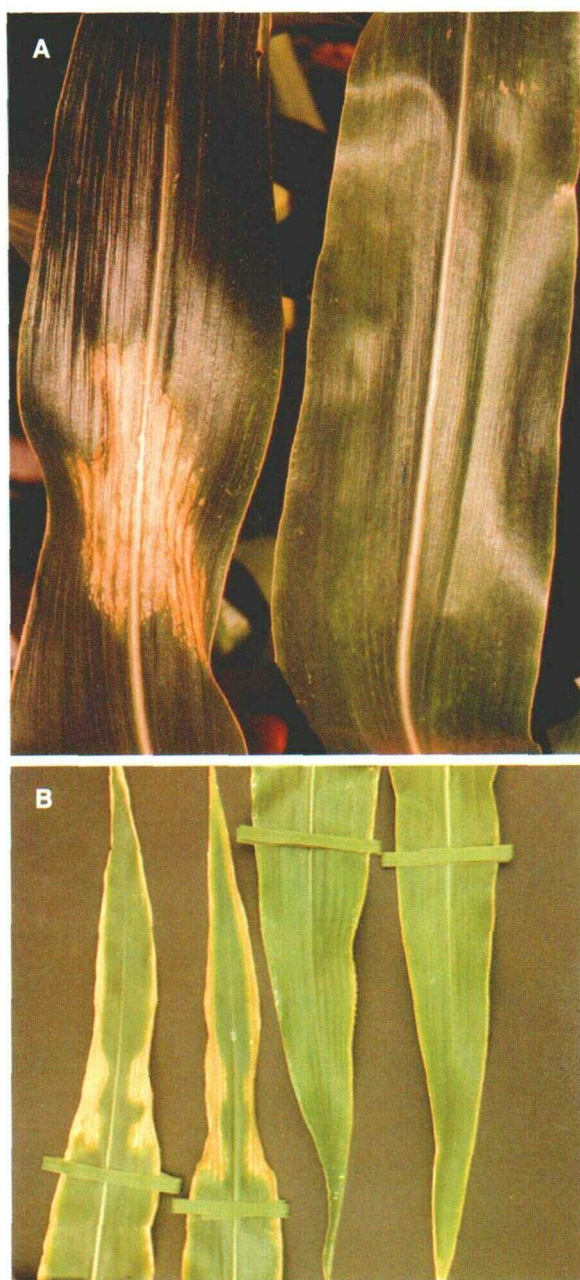


Figure 11. Basta Resistance in Transformed R₀ and R₁ Plants.

(A) A Basta[®] solution was applied to a large area ($\sim 4 \times 8$ cm) in the center of leaves of a nontransformed A188 \times B73 plant (left) and a transgenic R₀ E3/E4/E6 plant (right).

(B) Basta was also applied to leaves of four R₁ plants: two plants without *bar* (left) and two plants containing *bar* (right). The herbicide was applied to R₁ plants in 1-cm circles to four locations on each leaf, two on each side of the midrib. Photographs were taken 6 days after application.

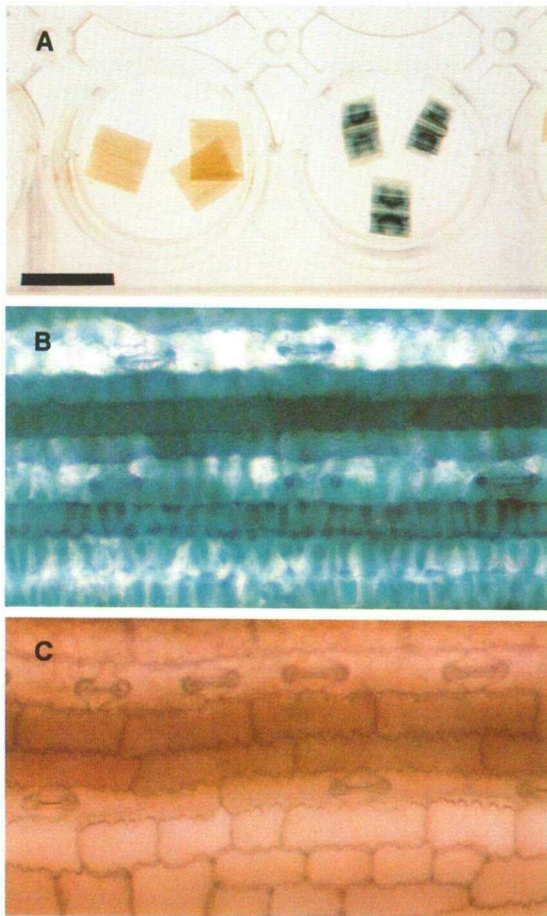


Figure 12. GUS Activity in Leaf Tissue of a Transgenic R_0 Plant.

(A) Histochemical determination of GUS activity in leaf tissue of a plant regenerated from cotransformed callus line Y13 (right) and a nontransformed tissue culture-derived plant (left). Bar = 1 cm.

(B) Light micrograph of the leaf segment from a Y13 plant shown in (A), observed in surface view under bright-field optics. GUS activity was observed in many cell types throughout the leaf tissue (magnification $\times 230$).

(C) Light micrograph as in (B) of control leaf.

Gordon-Kamm, unpublished results). The successful use of aminoglycosides, such as kanamycin, to identify transformants from cultured embryogenic cells may require optimization of several parameters, such as the stage of cell growth, concentration of antibiotic, and duration of exposure (Yang et al., 1988; Zhang et al., 1988; Lyznik et al., 1989). The endogenous resistance to kanamycin and G418 observed in many embryogenic maize cultures may be a contributing factor in the frequent recovery of highly resistant callus lines that do not contain *neo* (R.J. Daines,

unpublished results). In contrast to kanamycin and G418, we have found bialaphos to be an effective selective agent for embryogenic maize cultures.

In the experiments described in this paper, approximately one independent *bar*-transformed callus line was selected from the cells originating from one bombarded filter (Table 1). This corresponds to approximately one stable transformant recovered per 1000 cellular foci with transient GUS activity (or one transformant per 0.5 mL of packed cell volume). This estimation of stable transformation frequency is substantially lower than that determined for nonembryogenic BMS suspension cells (approximately 1 per 10 to 20 foci; Spencer et al., 1990). The difference in transformation frequency may be due to the fact that BMS cells are larger ($\sim 50 \mu\text{m}$) than the embryogenic cells ($\sim 20 \mu\text{m}$) and may incur less damage during bombardment. Alternatively, embryogenic suspensions represent a more heterogeneous cell population; there may be fewer cells competent to integrate DNA or to divide and form maintainable callus.

As reported for both maize (Tomes, 1990) and soybean (McCabe et al., 1988), microprojectile bombardment of meristems can result in chimeric tissue. In a cell culture selection system, chimeras could result from either cross-protection of nontransformed tissue by transformed tissue or from multiple transformation events within a cell population that give rise to a selected callus line. We have shown that, using bialaphos selection and microprojectile bombardment of embryogenic suspension cells, chimerism is not a problem. We have regenerated numerous plants from independent cell lines, and in all cases examined the plants tested had *bar* hybridization patterns and intensities identical to the parental callus. Inheritance of the introduced trait in the four fertile lines analyzed thus far (E2/E5, E10, R9, and R18) was consistent with the lack of chimeric callus and R_0 plants. Our data indicate that it is possible to transform single cells in heterogeneous maize cell cultures. These single transformed cells can divide, be selected, and give rise to maintainable callus lines capable of regeneration of fertile transgenic plants.

Phenotype and Fertility of Transgenic Plants

Certain phenotypic abnormalities associated with the plants regenerated from the SC82 callus lines were peculiar to an individual callus line and were consistent for all plants derived from the same callus line. This observation provides additional support for the single cell origin of these transformed callus lines. Distinctive phenotypes associated with a particular callus line may be due to somaclonal variation in the SC82 culture. Genetic and cytogenetic variation in plants regenerated from organogenic and friable, embryogenic maize tissue culture cells have been well documented (Armstrong and Phillips, 1988; Lee and Phillips, 1988). The variation observed in SC82 plants

probably arose from genetic changes present in the cells at the time of bombardment because distinctive phenotypic abnormalities associated with individual transformed cell lines were not observed using the same bombardment and selection protocol on a different culture, SC716. Almost all of the 219 R_0 plants regenerated from 10 independently transformed SC716 cell lines were similar in phenotype to nontransformed control plants. At least some of the fertility problems observed in SC82 R_0 plants were likely due to the culture itself and not to the transformation process. This is supported by the fact that most of the seeds recovered from the SC716 R_0 plants have not required embryo rescue. In the SC82 R_1 generation, the first generation after back-crossing to B73, phenotypic variability was expected. Variability was observed; however, no correlation between segregation of *bar* and specific phenotypic characteristics was evident.

Cotransformation

In *bar*-transformed callus, the cointegration frequency of the gene encoding GUS was 77%; coexpression frequency was 18% (Table 1). We have observed previously a coexpression frequency of 50% in BMS cells that were also bombarded with separate plasmids (Spencer et al., 1990). Klein et al. (1989) have recently reported a coexpression frequency of 100% in BMS cells bombarded with a single plasmid containing both the selected and nonselected genes. Depending on the mechanism(s) involved, coexpression frequencies in embryogenic cells may improve if both the selected and nonselected genes are on the same replicon.

GUS activity was determined by fluorometric analysis in six independent leaf extracts and was approximately 100-fold higher than control leaves. Although all fluorometric analyses were positive, some nonexpressing sectors of callus and leaf tissue were observed using the histochemical assay. Further analysis is needed to determine whether the nonexpressing sectors contain the nonselected gene and, if so, whether gene expression is blocked at the transcriptional or translational level. Alternatively, substrate availability may be a factor.

Conclusion

A great deal of our knowledge of genetics, plant physiology, and development derives from basic research of maize. Over the past decade, maize research has been constrained relative to dicotyledonous species for which transformation systems had become available. We now have shown that maize cells, including those recovered from cryopreserved cultures, can be genetically transformed and regenerated into fertile transgenic plants, and

that the foreign gene can be transmitted to progeny. This system provides a new, powerful tool for both the study of basic plant biology and the introduction of important, agronomic traits into one of the world's major crops.

METHODS

Plant Material

Friable, embryogenic type II callus (Armstrong and Green, 1985) was initiated from immature embryos (1.6 mm to 1.8 mm) excised from greenhouse-grown A188 \times B73 and A188 \times B84 plants. The callus was initiated and maintained at $\sim 10 \mu\text{E m}^{-2} \text{ sec}^{-1}$ light and 24°C on N6 medium (Chu et al., 1975) containing 2 mg/L glycine, 2.9 g/L L-proline, 100 mg/L casein hydrolysate, 13.2 mg/L dicamba or 1 mg/L 2,4-D, 20 g/L sucrose, pH 5.8, solidified with 2 g/L Gelgro (ICN Biochemicals, Cleveland, OH). Suspension cultures were initiated from independent callus initiations by placing approximately 1 g of callus tissue into 20 mL of liquid, modified MS medium (Murashige and Skoog, 1962) containing 0.25 mg/L thiamine, 2.9 g/L L-proline, 100 mg/L *myo*-inositol, 200 mg/L casein hydrolysate, 9.9 mg/L dicamba or 1 mg/L 2,4-D, 1.6 mg/L α -naphthaleneacetic acid, and 30 g/L sucrose, pH 6.0. Suspension cultures were maintained in liquid medium in the dark at 25°C on a rotary shaker (120 rpm) and were subcultured every 3 days to 7 days. After approximately 7 weeks, small cell aggregates began to predominate. To preferentially enrich for small clusters of cytoplasmically dense cells, suspension cultures were transferred by allowing dense cell clusters to settle to the bottom of the flask. One milliliter of packed cell volume (PCV) of the denser cell fraction and 4 mL of conditioned medium were transferred to fresh medium. Suspension culture SC82 was initiated from type II callus maintained on solidified N6-based culture medium for 3 months. SC82 cells were grown in liquid medium for approximately 4 months before bombardment (experiments 1 and 2). SC82 cells were also cryopreserved 5 months after suspension culture initiation, stored frozen for 5 months, thawed, and used for bombardment (experiment 6). Suspension culture SC716 was initiated from type II callus maintained 5 months in culture on solidified N6-based medium. SC716 cells were cultured in liquid medium for 5 months, cryopreserved for 8 months, thawed, and used 2 months later in bombardment experiments 4 and 5. SC94 was initiated from 10-month-old A188 \times B84 type II callus and cultured in liquid medium for 5 months before bombardment (experiment 3). Cell suspensions were cryopreserved using modifications of methods previously reported (Withers and King, 1979; Finkle et al., 1985). The cryoprotectants were added slowly to the suspension culture to give a final concentration of 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 mol wt), 0.23 M proline, and 0.23 M glucose, and then the mixture was cooled at 1°C/min to -35°C . After an isothermal period of 45 min, the samples were placed in liquid N_2 for storage. To reinstate suspension cultures from the cryopreserved material, cells were thawed rapidly (Withers and King, 1979) and pipetted onto plates containing feeder cells similar to those described by Rhodes et al. (1988). After presence of feeder cells for 1 week to 2 weeks, cells were reinoculated into liquid culture as described for the initiation of the suspension cultures.

Plant Expression Vectors

The construction of the vector encoding PAT (pDPG165) was described previously (Spencer et al., 1990) and the sequence of the *bar* gene used in these constructions was recently published (White et al., 1990). An additional vector encoding GUS, pDPG208, was used in these experiments. It was constructed using a 2.1-kb BamHI/EcoRI fragment from pAGUS1 (provided by J. Skuzeski, University of Utah) containing the coding sequence for GUS and the *nos* 3'-end from *Agrobacterium tumefaciens*. In pAGUS1 the 5'-noncoding and 5'-coding sequences for GUS were modified to incorporate the Kozak consensus sequence (Kozak, 1984) and to introduce a new HindIII restriction site 6 bp into the coding region of the gene (Skuzeski et al., 1990). The 2.1-kb BamHI/EcoRI fragment from pAGUS1 was ligated into a 3.6-kb BamHI/EcoRI fragment of a pUC19-based vector pCEV1 (provided by Calgene, Inc., Davis, CA). The 3.6-kb fragment from pCEV1 contains pUC19 and a 430-bp 35S promoter from cauliflower mosaic virus adjacent to the first intron from maize *Adh1*.

Microprojectile Bombardment

Plasmid DNA was precipitated in a 1:1 molar ratio onto tungsten particles (average diameter approximately 1.2 μ m, GTE Sylvania) using a procedure modified from Klein et al. (1987). The precipitation mixture included 1.25 mg of tungsten particles, 25 μ g of plasmid DNA, 1.1 M CaCl_2 , and 8.7 mM spermidine in a total volume of 575 μ L. After adding the components in the above order, the mixture was vortexed at 4°C for 10 min and centrifuged (500g) for 5 min, and 550 μ L of supernatant was decanted. From the remaining 25 μ L of suspension, 1- μ L aliquots were pipetted onto the macroprojectile for bombardment.

Cell suspensions used for microprojectile bombardment were generally rapidly growing, dispersed, heterogeneous populations. Within these suspensions, a range of cluster sizes and cell types (i.e., small, dense isodiametric cells; long vacuolated cells) was observed. Before bombardment, suspension culture cells were sieved through 1000- μ m stainless steel mesh. From the fraction of cell clusters passing through the sieve, approximately 0.5 mL of PCV was pipetted onto 5-cm filters (Whatman No. 4) and vacuum filtered in a Buchner funnel. The filters were transferred to Petri dishes containing three 7-cm filters (Whatman No. 4) moistened with 2.5 mL of suspension culture medium. The tissue was positioned approximately 5 cm below the macroprojectile stopping plate, and a 100- μ m mesh stainless steel screen was placed halfway between the stopping plate and the tissue to aid in dispersion of the tungsten particles. Bombardments were performed as described previously (Spencer et al., 1990).

Transformant Selection

Immediately after bombardment, cells were gently removed from the support filter and resuspended in modified MS medium in the absence of bialaphos. After 7 days to 14 days in liquid medium, approximately 0.5 mL of PCV was vacuum-filtered onto 7-cm filters (Whatman No. 4) using a Buchner funnel. Filters were transferred to N6 maintenance medium (see above) containing 5.5 g/L Sea Kem agarose (FMC Corp.) and 1 or 3 mg/L of filter-sterilized bialaphos (Meiji Seika Kaisha, Ltd., Yokohama, Japan). Cells were transferred on filters to fresh selection medium every

7 days. After 3 weeks to 4 weeks, most of the tissue plated on 1 mg/L bialaphos was removed from the filters as discrete clumps (~5 mm in diameter) and placed directly on medium containing 1 mg/L bialaphos. Tissue on 3 mg/L bialaphos and some of the tissue on 1 mg/L bialaphos was cultured without removing the tissue from the original selection filter. Resistant colonies were isolated 6 weeks to 7 weeks post-bombardment and maintained as individual cell lines on medium containing 1 mg/L bialaphos.

Enzyme Assays

PAT activity determinations were performed using a modified thin-layer chromatographic assay (Spencer et al., 1990). Approximately 25 μ g of protein was used per assay. GUS activity was assessed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide as described (Jefferson, 1987); tissue was scored for blue cells 18 hr to 24 hr after addition of substrate. Fluorometric analysis was performed as described by Jefferson (1987) using 4-methylumbelliferyl glucuronide.

DNA Gel Blot Analysis

Genomic DNA was isolated using a modification of the procedure of Shure et al. (1983). Approximately 1 g of callus tissue was ground to a fine powder in liquid N_2 using a mortar and pestle. Powdered tissue was mixed thoroughly with 4 mL of extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% sarcosine). Tissue/buffer homogenate was extracted with 4 mL of phenol/chloroform. The aqueous phase was separated by centrifugation, passed through Miracloth, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2, and an equal volume of isopropyl alcohol. The precipitate was washed with 70% ethanol and resuspended in 200 μ L to 500 μ L of TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0). Genomic DNA was digested with a threefold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC Corp.), and transferred (Southern, 1975) to Nytran (Schleicher & Schuell) using 10 \times SCP. (20 \times SCP is 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA.) Filters were prehybridized at 65°C in 6 \times SCP, 10% dextran sulfate, 2% sarcosine, and 500 μ g/mL heparin (Chomet et al., 1987) for 15 min. Filters were hybridized overnight at 65°C in 6 \times SCP containing 100 μ g/mL denatured salmon sperm DNA and ^{32}P -labeled probe. The 0.6-kb SmaI fragment from pDPG165 and the 1.8-kb BamHI/EcoRI fragment from pCEV5 (Spencer et al., 1990) were used in random priming reactions (Feinberg and Vogelstein, 1983; Boehringer Mannheim) to generate labeled probes for detecting sequences encoding PAT or GUS, respectively. Filters were washed in 2 \times SCP, 1% SDS at 65°C for 30 min and visualized by autoradiography using Kodak X-AR5 film. Before rehybridization with a second probe, the filters were boiled for 10 min in distilled H_2O to remove the first probe and then prehybridized as described above.

Regeneration

Fertile transgenic plants were regenerated by transferring embryogenic callus to MS medium containing 0.25 mg/L 2,4-D and 10.0 mg/L 6-benzylaminopurine. Tissue was maintained on this me-

dium for approximately 2 weeks and subsequently transferred to MS medium without hormones (Shillito et al., 1989). Shoots that developed after 2 weeks to 4 weeks on hormone-free medium were transferred to MS medium containing 1% sucrose and solidified with 2 g/L Gelgro® in Plant Con® containers where rooting occurred. Alternative regeneration routes using media containing high cytokinin/auxin ratios were also successful. More mature embryogenic callus could be regenerated on N6 (Chu et al., 1975) medium containing 6% sucrose and no hormones (Armstrong and Green, 1985) for 2 weeks, followed by transfer to MS medium without hormones as described above. Regeneration was performed at 25°C under fluorescent lights (250 $\mu\text{E m}^{-2} \text{sec}^{-1}$). After approximately 2 weeks, developing plantlets were transferred to soil, hardened off in a growth chamber (85% relative humidity, 600 ppm CO_2 , 350 $\mu\text{E m}^{-2} \text{sec}^{-1}$), and grown to maturity in either a growth chamber or the greenhouse.

Embryo Rescue

Developing embryos were excised from surface disinfected kernels 10 days to 20 days post-pollination and cultured on medium containing MS salts, 2% sucrose, and 5.5 g/L Sea Kem agarose. Large embryos (>3 mm) were germinated directly on the medium described above. Smaller embryos were cultured for approximately 1 week on the above medium containing 10^{-5} M abscisic acid and transferred to hormone-free medium for germination. Several embryos became bacterially contaminated; these embryos were transferred to medium containing 300 $\mu\text{g/mL}$ cefoxitin. Developing plants were subsequently handled as described for regeneration of R_0 plants.

Herbicide Application

The herbicide formulation used, Basta TX®, contains 200 g/L glufosinate, the ammonium salt of phosphinothricin. Young leaves were painted with a 2% Basta solution (v/v) containing 0.1% (v/v) Tween 20. The prescribed application rate for this formulation is 0.5% to 1%.

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